CCXXXIII. THE MECHANISM OF THE REACTION OF SUBSTRATES WITH MOLECULAR OXYGEN. II.

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I. THE EFFECT OF CYANIDE ON OXIDATIONS BY BAKER'S YEAST.

THERE is an extensive literature dealing with the effect of cyanide on the total respiration of various cells. Scanty information is available, however, on the effect of cyanide on the oxidation of known substrates by intact cells.

If a particular oxidation *in vivo* is not cyanide-sensitive, it is reasonable to conclude that this oxidation is not catalysed by cytochrome and the indophenol oxidase. On the other hand a positive cyanide effect can mean either that the cytochrome system or that some other cyanide-sensitive system is involved. That is to say cyanide inhibition is not a specific indicator of cytochrome catalysis. Complete proof of the working of the cytochrome system requires the demonstration not only of inhibition by cyanide but also of inhibition by H₂S, CO and NaN₃, and of the reversal of CO inhibition by light of a definite wave-length. With the exception of Warburg's [1927] study of the oxidation of alcohol, glucose and acetic acid by baker's yeast, there has been no thorough analysis of the effects of various inhibitors on the oxidations of particular substrates.

Experimental. In order to reduce the residual respiration to a minimum, Delft yeast was washed twice with 15 volumes of tap water, suspended in *M*/5 phosphate buffer *p*_H 7·2 and aerated vigorously for 6 hours. The yeast was

Table I. *The effect of cyanide (final concentration M/600) on the metabolism of baker's yeast.*

Yeast (ml.)	1	1	1	1	1	1	1	1	1	1	1	1
KCN, <i>M</i> /100	—	0·5	—	0·5	—	0·5	—	0·5	—	0·5	—	0·5
Lactate, 10 %	—	—	0·5	0·5	—	—	—	—	—	—	—	—
Hexosemonophosph., 0·35 <i>M</i>	—	—	—	—	0·3	0·3	—	—	—	—	—	—
Hexosediphosph., <i>M</i> /3	—	—	—	—	—	—	0·3	0·3	—	—	—	—
α-Glycerophosphate, <i>M</i> /10	—	—	—	—	—	—	—	—	0·5	0·5	—	—
Glucose, 1 <i>M</i> *	—	—	—	—	—	—	—	—	—	—	0·5	0·5
Distilled water	2	1·5	1·5	1	1·7	1·2	1·7	1·2	1·5	1	1·5	1
O ₂ uptake (μl./30 min.)	65·5	26·4	570	54	298	25	123	41	149	56·5	306	65·5
% inhibition	—	—	—	95	—	100	—	75	—	64	—	84

* In the glucose experiments, the manometers were filled with pure oxygen instead of air in order to minimise fermentation and the production of CO₂.

Table II. *The effect of cyanide (final concentration M/600) on the oxidation of alcohol by Delft yeast.*

Yeast (ml.)	0·5	0·5	0·5	0·5
KCN, <i>M</i> /100	—	0·5	—	0·5
Alcohol, <i>M</i> /10	—	—	0·5	0·5
Distilled water	2·5	2	2	1·5
O ₂ uptake (μl./30 min.)	85	90	405	52
% inhibition	—	—	—	100

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centrifuged and resuspended in fresh phosphate buffer. Tables I and II show the effects of cyanide on the oxidations of lactate, hexosemonophosphate, hexosediphosphate, α -glycerophosphate, glucose and alcohol. $M/600$ cyanide was used since trial experiments showed that this concentration was not high enough for toxic action and not low enough for submaximum inhibition. The results show almost complete inhibition of all the systems studied except the α -glycerophosphate which was inhibited to the extent of only 64 % in this experiment. The percentage inhibition is calculated from the following formula:

$$\% \text{ inhibition} = 100 - \frac{\text{oxygen uptake of substrate in cyanide} - \text{oxygen uptake of blank in cyanide}}{\text{oxygen uptake of substrate} - \text{oxygen uptake of blank}}.$$

There is a certain amount of variability in the magnitude of the cyanide inhibition, but experiment has shown that there is a rule governing this variability. If for some reason (as yet unknown) a particular dehydrogenase is not active in a given sample of yeast, the small increase over the blank uptake is either very slightly or not at all affected by addition of cyanide. When, however, the enzyme is found active in other samples of yeast, the large increase over the blank reaction is largely eliminated by addition of cyanide. No oxidation has yet been found which under proper conditions was not largely inhibited by $M/600$ cyanide.

The effect of cyanide on the oxidations of yeast suspended in $M/5 \text{ KH}_2\text{PO}_4$ was tried and the results were found to be similar to those in Table I.

The oxidations of hypoxanthine, succinate, formate, acetaldehyde and malate by Delft yeast were found to be insufficiently rapid to permit accurate measurement of the effect of cyanide.

The cyanide inhibitions observed raise the following questions. If the oxidation of hexosemonophosphate or hexosediphosphate involves yellow pigment catalysis, why is the catalysis cyanide-sensitive *in vivo* when there is no effect of cyanide on the isolated dehydrogenase system? If the cyanide-sensitivity *in vivo* is taken to mean the operation of a cytochrome catalysis, why cannot the hexosephosphate dehydrogenase systems couple *in vitro* with cytochrome and the indophenol oxidase? Warburg [1928], in referring to the question of the number of "Atmungsfermente", makes a distinction between experiments on isolated systems and on living cells, and states that effects which obtain with the one do not necessarily obtain with the other. This may be the answer to the dilemma arising from the cyanide experiments, though it is by no means certain.

All the evidence points to the alcohol dehydrogenase of baker's yeast reacting with molecular oxygen by way of cytochrome and the "Atmungsferment" [Warburg, 1927]. The oxidation is sensitive both to CO and cyanide. Further, the partition constant for the oxidation of alcohol in presence of different mixtures of CO and O₂ is identical within the limits of experimental error with that of the "Atmungsferment". Adler and Euler [1934] have demonstrated that the alcohol dehydrogenase *in vitro* can react with molecular oxygen through the intermediation of yellow pigment, and they have concluded that the dehydrogenase normally functions in association with "Flavinenzym". It appears, however, that this catalysis may be artificial and may not take place in the living cell.

II. THE METABOLISM OF BOTTOM YEAST.

This organism offers excellent material for testing some aspects of the theories of cellular respiration. It is normally anaerobic and contains a relatively large quantity of yellow pigment (20 mg. photoderivative/kg. dry weight,

Warburg and Christian, [1933]), but no cytochrome or indophenol oxidase. According to Windisch [1932] the Q_{O_2} of bottom yeast varies from 1 to 4 whilst the $Q_{CO_2}^N$ lies between the limits of 80 and 120.

The question arose whether the low Q_{CO_2} of bottom yeast is due to a deficiency in some important component of the aerobic mechanism rather than to the complete absence of respiratory systems. The following experiments were carried out with a view to elucidating the causes of the low Q_{O_2} of bottom yeast.

EXPERIMENTAL.

Bottom yeast was obtained from (1) a Belgian brewery and (2) Barclay, Perkins and Co. Ltd., London. The yeast was washed by decantation at least 5 times with 20 volumes of tap water, and finally suspended in $M/4$ phosphate buffer at p_H 7.2.

Tables III–VIII summarise the experiments dealing with the metabolism of the English and Belgian samples of bottom yeast. The most active dehydrogenase systems are the hexosemonophosphate, hexosediphosphate, lactate, glucose and pyruvate. The effects of addition of Warburg coferment and pyocyanine show clearly that the missing components of the respiratory complement are (1) coenzyme and (2) intermediary carrier. By supplying the deficient elements, the oxygen uptake can be increased as much as tenfold. Pyocyanine is far more efficient as a carrier than methylene blue. Flavin has either no effect or a slight inhibitory effect. It is noteworthy that cozymase is not equivalent to Warburg coferment in increasing the velocity of the oxidation of hexosemonophosphate. This observation agrees with our experience of the unequal effects of cozymase and of Warburg coferment on the isolated dehydrogenase system.

Table III. *Metabolism of bottom yeast (Belgian). Oxidation of lactic acid.*

Yeast (ml.)	0.5	0.5	0.5	0.5	0.5
Lactate, 10%	—	0.5	0.5	0.5	0.5
Methylene blue, 1/5000	—	—	0.5	—	—
Pyocyanine, 1/5000	—	—	—	0.5	0.5
Cozymase, 33 units/ml.	—	—	—	—	0.5
Distilled water	2.5	2	1.5	1.5	1
O_2 uptake (μ l./30 min.)	26.3	43.2	61	126	108

Table IV. *Metabolism of bottom yeast (English). Oxidation of hexosemonophosphate.*

Yeast (ml.)	1	1	1	1	1	1	1
Hexosemonophosph., 0.35 M	—	0.3	—	0.3	0.3	0.3	0.3
Warburg coferment	—	—	0.5	0.5	0.5	—	0.5
Pyocyanine, 1/5000	—	0.5	0.5	—	0.5	—	—
Flavin, 10 γ /ml.	—	—	—	—	—	—	0.5
Distilled water	2	1.5	1.2	1.5	1.2	0.7	1.7
O_2 uptake (μ l./30 min.)	28	41	154	52	77	172	44

Table V. *Metabolism of bottom yeast (Belgian). Oxidation of hexosemonophosphate.*

Yeast (ml.)	1	1	1	1	1
Hexosemonophosph., 0.35 M	0.3	0.3	0.3	0.3	0.3
Warburg coferment	—	—	0.5	0.5	—
Pyocyanine, 1/5000	—	0.5	0.5	—	0.5
Cozymase, 80 units/ml.	—	—	—	—	0.5
Distilled water	1.7	1.2	0.7	1.2	0.7
O_2 uptake (μ l./30 min.)	49.5	66.5	307	82.0	61.5

Table VI. *Metabolism of bottom yeast (English). Oxidation of hexosediphosphate.*

Yeast (ml.)	1	1	1	1	1
Hexosediphosph., <i>M</i> /3	—	0.3	0.3	—	0.3
Warburg coferment	0.5	0.5	0.5	—	0.5
Pyocyanine, 1/5000	—	—	0.5	—	—
Flavin, 10γ/ml.	—	—	—	—	0.5
Distilled water	1.5	1.2	0.7	2.0	0.7
O ₂ uptake (μl./30 min.)	52	57	169	28	53

Table VII. *Metabolism of bottom yeast (Belgian). Oxidation of glucose, α-glycerophosphate, succinate, pyruvate, formate and alcohol.*

Yeast (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pyocyanine, 1/5000	—	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glucose, <i>M</i>	—	—	0.5	—	—	—	—	—
α-Glycerophosphate, <i>M</i> /10	—	—	—	0.5	—	—	—	—
Succinate, 10%	—	—	—	—	0.5	—	—	—
Formate, <i>M</i> /10	—	—	—	—	—	0.5	—	—
Alcohol, <i>M</i> /10	—	—	—	—	—	—	0.5	—
Pyruvate, <i>M</i> /10	—	—	—	—	—	—	—	0.5
Distilled water	2.5	2	1.5	1.5	1.5	1.5	1.5	1.5
O ₂ uptake (μl./30 min.)	26.3	28	107	44	53	42	65	116.5

Table VIII. *Metabolism of bottom yeast (Belgian). The effect of *M*/600 cyanide on the pyocyanine catalysis of the oxidation of hexosemonophosphate.*

Yeast (ml.)	0.5	0.5	0.5
Hexosemonophosph., 0.35 <i>M</i>	—	0.3	0.3
Warburg coferment	0.5	0.5	0.5
Pyocyanine, 1/5000	—	0.5	0.5
KCN, <i>M</i> /100	—	—	0.5
Distilled water	2.0	1.2	0.7
O ₂ uptake (μl./30 min.)	18.0	286	198

The enormous increase in respiration occasioned by pyocyanine is difficult to reconcile with the presence of relatively large amounts of yellow pigment in bottom yeast. The probable explanation is that yellow pigment is normally not concerned in the oxidations of hexosemonophosphate, hexosediphosphate and glucose. Probably the pigment is not accessible *in vivo* to these three dehydrogenase systems. Green *et al.* [1934] showed that yellow pigment was about three-fold as efficient as pyocyanine in catalysing the oxidation of hexosemonophosphate by the dehydrogenase system isolated from bottom yeast. Yet *in vivo* the oxidation of hexosemonophosphate in absence of added carrier proceeds at a negligible velocity. If the oxidation in the intact cell involved the yellow pigment, the addition of pyocyanine should have had little effect.

M/600 cyanide inhibits the pyocyanine catalysis of the oxidation of hexosemonophosphate by 31%. *In vitro* *M*/200 cyanide increases the velocity of oxygen uptake of the hexosemonophosphate enzyme system by 36% [Euler and Adler, 1934]. It appears therefore that enzymes in the intact cell are more cyanide-sensitive than in cell-free solution and that this cyanide-sensitivity in some cases may have nothing to do with haematin catalysis.

III. COUPLING OF DEHYDROGENASES WITH THE SUCCINOXIDASE-CYTOCHROME-INDOPHENOL OXIDASE SYSTEM.

In Part I of this series, the theory of Goszy and Szent-Györgyi [1934], attributing catalytic properties to the succinic-fumaric system, was discussed in detail. Experiments are now presented showing that *in vitro* the succinic-fumaric

system cannot act as an intermediary link between cytochrome and dehydrogenases. The method of testing was simply to compare the oxygen uptake of a solution containing dehydrogenase, substrate, indophenol oxidase and cytochrome with and without the addition of succinate equivalent to 22 μ l. O_2 . If the succinate is constantly regenerated according to the theory, then the increase in the oxygen uptake should be many times that for the complete oxidation of the added succinate. Table IX shows that the differences in oxygen uptake

Table IX. *The coupling of dehydrogenase systems with the succinoxidase-cytochrome-indophenol oxidase system.*

Enzyme (ml.)	α -glycero-phosphate		Formate		Glucose		Hexosemono-phosphate	
	1	1	1	1	1	1	1	1
*Succinate, $0.14 \times 10^{-5} M$	0.2	—	0.2	—	0.2	—	0.2	—
Indophenol oxidase	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cytochrome, $3 \times 10^{-4} M$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
α -Glycerophosphate, $M/10$	—	0.5	0.5	—	—	—	—	—
Formate, 10 %	—	—	—	0.5	0.5	—	—	—
Glucose, M	—	—	—	—	—	0.5	0.5	—
Hexosemonophosph., $0.35 M$	—	—	—	—	—	—	—	0.5
Distilled water	1.8	0.5	0.3	0.5	0.3	0.5	0.3	0.5
O_2 uptake (μ l./30 min.)	41	75	90	104	121	91	103	48

* 0.2 ml. succinate is equivalent to 22.4 μ l. O_2 .

with and without succinate in the cases of the α -glycerophosphate, formate, glucose and hexosemonophosphate dehydrogenases are never more than 24 μ l. O_2 . In other words, the reactions between activated fumaric acid and the four substrates tried cannot take place directly. The possibility remains that in the cell some carrier links fumarate and the substrate. The experiments of Green *et al.* [1934] have shown that none of the known natural carriers can function in carrier-linked reactions between dehydrogenase systems. But there may be as yet unknown carriers which perform the coupling in the cell.

IV. INDOPHENOL OXIDASE ACTIVITY AND THE Q_{O_2} OF RAT TISSUES.

If the cytochrome-indophenol oxidase system is the principal respiratory system of animal tissues, there should exist a proportionality between the intensity of respiration and the amount of indophenol oxidase. Keilin [1925; 1929; 1930] maintained that qualitatively this relation does hold. The following experiment was carried out to test the proportionality quantitatively. Fresh tissues were weighed, thoroughly minced, washed twice with 0.9 % NaCl and ground to a homogeneous suspension in a known volume of $M/5$ phosphate buffer p_H 7.2. The $Q_{\text{indophenol oxidase}}$ was calculated on the basis of the original dry weight of the tissue prior to washing with salt solution. Trial experiments showed that no oxidase was washed out from the tissue by the salt solution. Table X contains the comparison of the Q_{O_2} and the $Q_{\text{indophenol oxidase}}$. Brain with the lowest Q_{O_2} of the four tissues studied has the highest $Q_{\text{indophenol oxidase}}$. The other three tissues have comparable Q_{O_2} and $Q_{\text{indophenol oxidase}}$ values. It is noteworthy that the Q_{O_2} and the $Q_{\text{indophenol oxidase}}$ are of the same order of magnitude. In other words there is approximately sufficient indophenol oxidase to account for the greater part of respiration. The agreement is not perfect but it is worth mentioning that the indophenol oxidase activity was measured in cell-free suspension whereas the Q_{O_2} was measured with the intact slice. In general there is a falling off in the activity of oxidation enzymes with extraction from the cell.

Table X. *Comparison of indophenol oxidase activity and Q_{O_2} of rat tissues.*

$Q_{\text{indophenol oxidase}}$ - O_2 uptake in $\mu\text{l./mg. dry weight/hour}$, in the presence of *p*-phenylenediamine. (3 mg.)

	$Q_{\text{indophenol oxidase}}^*$	Q_{O_2}	Ratio
Liver	10.2	20	1.9
Heart	14.3	22	1.5
Kidney	25.5	40	1.6
Brain	33.5	14	0.4

* The figures for the Q_{O_2} were kindly given to us by Dr H. A. Krebs from unpublished work, and represent the maximum respiration of the various tissues.

V. THE ASSOCIATION OF THE INDOPHENOL OXIDASE WITH DISCRETE PARTICLES.

Numerous attempts were made to purify and concentrate the indophenol oxidase from heart muscle but no success was attained. Experiment showed that the heart muscle preparation represents a suspension of fine particles with which the enzyme is intimately associated. No means has yet been found of separating the enzyme from the particles. Tables XI and XII show the effect of

Table XI. *The effect of high-speed centrifuging on the activity of the indophenol oxidase preparation of sheep's heart.*

	Before centrifuging	1 hour at 14,000 r.p.m.
Enzyme (ml.)	1	1
<i>p</i> -Phenylenediamine, 10 mg./ml.	0.4	0.4
Distilled water	1.6	1.6
O_2 uptake ($\mu\text{l./30 min.}$)	277	45

Table XII. *The effect of high-speed centrifuging on the activity of the indophenol oxidase and succinoxidase preparations of sheep's heart.*

	Before centrifuging		45 min. at 14,000 r.p.m.	
Enzyme (ml.)	1	1	1	1
<i>p</i> -Phenylenediamine, 10 mg./ml.	0.5	—	0.5	—
Succinate, 10 %	—	0.5	—	0.5
Distilled water	1.5	1.5	1.5	1.5
O_2 uptake ($\mu\text{l./30 min.}$)	108	163	52	38

high-speed centrifuging on the activity of the indophenol oxidase preparation. It is clear that centrifuging, if continued long enough, will remove all the activity of both the succinic and indophenol oxidases.

VI. IS THE YELLOW PIGMENT A CARRIER OR AN ENZYME?

The theory of Wagner-Jauregg *et al.* [1934; Wagner-Jauregg, 1935] and of Adler and Euler [1934; 1935] that the yellow pigment works in conjunction with the dehydrogenase in reducing flavin, methylene blue or oxygen can be tested as follows. If the yellow pigment functions enzymically, then supplying flavin to the flavin-enzyme system should increase the oxygen uptake, whereas if the yellow pigment functions purely as an intermediary carrier between the substrate and oxygen, the addition of flavin should have no effect since adding flavin is equivalent to adding an oxidising agent which competes with oxygen for reduced yellow pigment. Tables XIII-XVI show that in no case does addition of lactoflavin increase the yellow pigment catalysis, and that in most cases

Table XIII. *The combined effect of yellow pigment and flavin on the glucose dehydrogenase of liver.*

Enzyme (ml.)	1	1	1	1
Cozymase, 400 units/ml.	0.5	0.5	0.5	0.5
Glucose, 3 M	0.5	0.5	0.5	0.5
Yellow pigment, 20 %	—	0.5	—	0.5
Flavin, 10 γ /ml.	—	—	0.5	0.5
Distilled water	1	0.5	0.5	0
O ₂ uptake (μ l./30 min.)	128	146	134	162
% increase	—	14	—	27

Table XIV. *The combined effect of yellow pigment and flavin on the hexosemonophosphate dehydrogenase. (Horse corpuscles.)*

Enzyme (ml.)	1.5	1.5	1.5	1.5
Warburg coferment	0.6	0.6	0.6	0.6
Hexosemonophosph., 0.35 M	0.3	0.3	0.3	0.3
Yellow pigment, 20 %	—	0.3	—	0.3
Flavin, 1 mg./10 ml.	—	—	0.3	0.3
Distilled water	0.6	0.3	0.3	—
O ₂ uptake (μ l./30 min.)	36.6	185	61.5	176
% increase	—	400	—	380

Table XV. *The combined effect of yellow pigment and flavin on the malic dehydrogenase. (Heart.)*

Enzyme (ml.)	1	1	1	1
Cozymase, 400 units/ml.	1	1	1	1
Malate, M	0.4	0.4	0.4	0.4
Yellow pigment, 20 %	—	0.3	—	0.3
Flavin, 100 γ /ml.	—	—	0.3	0.3
Distilled water	0.6	0.3	0.3	—
O ₂ uptake (μ l./30 min.)	38	72	51	50
% increase	—	90	37	37

Table XVI. *The combined effect of yellow pigment and flavin on the hexosediphosphate dehydrogenase. (Horse corpuscles.)*

Enzyme (ml.)	1.5	1.5	1.5	1.5
Warburg coferment	0.6	0.6	0.6	0.6
Hexosediphosph., M/3	0.3	0.3	0.3	0.3
Yellow pigment, 20 %	—	0.3	—	0.3
Flavin, 1 mg./10 ml.	—	—	0.3	0.3
Distilled water	0.6	0.3	0.3	—
O ₂ uptake (μ l./30 min.)	46	54	56	57.5

flavin has a definite inhibitory effect. There is therefore no basis for ascribing enzymic properties to the yellow pigment.

We are grateful to Priv. Doz. Dr Wagner-Jauregg for a sample of pure crystalline lactoflavin. No difference was found between the behaviour of our crude and his pure material.

SUMMARY.

1. *M*/600 cyanide inhibits almost completely the oxidations of lactate, hexosemonophosphate, hexosediphosphate, α -glycerophosphate, glucose and alcohol by actively respiring baker's yeast. When any of these dehydrogenase systems are relatively weak in a given sample of yeast, as shown by the fact that the addition of substrate increases only slightly the blank oxygen uptake, *M*/600 cyanide is found to have little effect.

2. The oxygen uptake of bottom yeast in presence of hexosemonophosphate, hexosediphosphate, glucose, lactate and pyruvate is greatly increased by addition

of pyocyanine. Warburg coferment from horse corpuscles augments the pyocyanine effect in the cases of the oxidation of hexosemonophosphate and hexosediphosphate.

3. The α -glycerophosphate, formate, glucose and hexosemonophosphate dehydrogenases cannot react with cytochrome through the intermediation of the succinic-fumaric system.

4. The $Q_{O_2}/Q_{\text{indophenol oxidase}}$ ratio of rat liver, heart and kidney is fairly constant (1.5–1.9). However, rat brain has the lowest Q_{O_2} and the highest $Q_{\text{indophenol oxidase}}$ of the four tissues studied. The $Q_{\text{indophenol oxidase}}$ values are sufficiently high to account for the bulk of the respiration of these tissues.

5. The indophenol oxidase is associated with macroscopic particles which can be sedimented by centrifuging at high speed.

6. Flavin either inhibits or has no effect on the yellow pigment catalysis of the enzymic oxidation of glucose, hexosemonophosphate, hexosediphosphate and malate.

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